Clinical Science (2017) **131** 653–671
DOI: 10.1042/CS20160732

PORTLAND PRESS

Research Article

Annexin II-binding immunoglobulins in patients with lupus nephritis and their correlation with disease manifestations

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Annexin II on mesangial cell surface mediates the binding of anti-dsDNA antibodies and consequent downstream inflammatory and fibrotic processes. We investigated the clinical relevance of circulating annexin II-binding immunoglobulins (Igs) in patients with severe proliferative lupus nephritis, and renal annexin II expression in relation to progression of nephritis in New Zealand Black and White F1 mice (NZBWF1/J) mice. Annexin II-binding Igs in serum were measured by ELISA. Ultrastructural localization of annexin II was determined by electron microscopy. Seropositivity rates for annexin II-binding IgG and IgM in patients with active lupus nephritis were significantly higher compared with controls (8.9%, 1.3% and 0.9% for annexin II-binding IgG and 11.1%, 4.0% and 1.9% for annexin II-binding IgM for patients with active lupus nephritis, patients with non-lupus renal disease and healthy subjects respectively). In lupus patients, annexin II-binding IgM level was higher at disease flare compared with remission. Annexin II-binding IgG and IgM levels were associated with that of anti-dsDNA and disease activity. Annexin II-binding IgG and IgM levels correlated with histological activity index in lupus nephritis biopsy samples. In NZBWF1/J mice, serum annexin II-binding IgG and IgM levels and glomerular annexin II and p11 expression increased with progression of active nephritis. Annexin II expression was present on mesangial cell surface and in the mesangial matrix, and co-localized with electron-dense deposits along the glomerular basement membrane. Our results show that circulating annexin II-binding IgG and IgM levels are associated with clinical and histological disease activity in proliferative lupus nephritis. The co-localization of annexin II and p11 expression with immune deposition in the kidney suggests pathogenic relevance.

Introduction

Systemic lupus erythematosus (SLE) is a prototypic autoimmune disease characterized by a breakdown of immune tolerance and production of autoantibodies, the majority of which are directed against chromatin material [1]. Although SLE has a wide spectrum of clinical manifestations, kidney involvement, termed lupus nephritis, affects up to 60% of SLE patients and is an important cause of morbidity and mortality [2]. Up to 30% of patients with lupus nephritis progress to end-stage renal disease. Lupus nephritis is initiated by the deposition of anti-dsDNA antibody (Ab)-containing immune complexes in the kidney parenchyma, which results in complement activation, mesangial and endocapillary proliferation, immune cell infiltration, and induction of inflammatory and fibrotic processes. Anti-dsDNA antibodies can be detected in approximately 90% of lupus patients compared with less than 0.5% in healthy subjects or patients with other autoimmune diseases [1,3]. The importance of

Received: 19 September 2016
Revised: 7 February 2017
Accepted: 8 February 2017

Accepted Manuscript online: 9 February 2017
Version of Record published: 28 March 2017

Clinical Science (2017) **131** 654–671
DOI: 10.1042/CS20160732

anti-dsDNA antibodies in pathogenesis is exemplified by the temporal association of rising anti-dsDNA Ab titres during flare, their presence in glomerular immune deposits in patients and mice with active lupus nephritis and the observation that many features of lupus nephritis can be replicated in non-autoimmune mice following their inoculation with the transgene encoding the secreted form of an IgG anti-dsDNA Ab [4–9].

Anti-dsDNA antibodies deposit in the kidney parenchyma either through direct binding to cross-reactive antigens on the surface of resident renal cells or through interaction with chromatin material entrapped in the glomerular basement membrane (GBM) or surrounding structures [6,10–13]. We previously demonstrated that anti-dsDNA antibodies could bind directly to mesangial cell surface annexin II and then became internalized, and this was associated with induction of mitogen-activated protein kinase (MAPK) and phosphatidylinositol 4,5-bisphosphate 3-kinase/protein kinase B (PI3K/AKT) signalling pathways and increased interleukin-6 (IL-6) secretion [6]. The up-regulation of glomerular annexin II expression in lupus nephritis and its co-localization with IgG and complement 3 (C3) suggest that this pathway is of pathogenic significance [6]. We and other investigators reported the presence of annexin II-binding IgG in patients with lupus, and part of this could be attributed to anti-dsDNA antibodies [6,14].

Annexin II, also known as lipocortin II, calpactin I and heavy polypeptide (p36), is a 36 kDa calcium-dependent phospholipid-binding protein expressed by many cell types including mesangial cells, endothelial cells and monocytes [6,15,16]. Annexin II plays important roles in plasmin activation, endothelial cell activation, neo-angiogenesis, cell proliferation, membrane trafficking and apoptosis [16–20]. p11, also known as S100A10 or calpactin I light chain, is a ligand of annexin II, and often forms a heterotetrameric complex with annexin II [21,22]. Unlike other S100 proteins, p11 is insensitive to calcium, serves as an effector protein for annexin II-mediated fibrinolysis and regulates exocytosis, endocytotosis and signal transduction, the latter mediated through its ability to increase serotonin receptor expression [21,23,24]. Autoantibodies to annexin II have been detected in patients with antiphospholipid syndrome (APS), rheumatoid arthritis and to a lesser extent in SLE patients [25,26]. While the titre of anti-dsDNA antibodies correlates with disease activity in most patients, the level measured using commercial assays could include different subsets with variable pathogenic relevance. In contrast, the clinical or pathogenic significance of anti-annexin II antibodies in lupus nephritis patients has not been investigated. In the present study, we investigated annexin II-binding IgG, IgM and IgA in the serum of patients with severe proliferative lupus nephritis and examined their association with disease parameters. We also explored the relationship of annexin II expression with electron-dense immune deposits in the kidney. In addition, we studied the intra-glomerular expression of annexin II and p11 as nephritis progressed in New Zealand Black and White F1 (NZBWF1/J) mice. Our data demonstrate that serum annexin II-binding IgG and IgM levels are likely of clinical significance since they correlate with histological activity index in the kidney biopsy and also with the level of anti-dsDNA in a significant proportion of patients. The co-localization of annexin II and p11 expression with electron-dense immune deposits in ultrastructural studies in NZBWF1/J mice also suggests that annexin II and its binding immunoglobulins (Igs) are of pathogenic relevance.

Materials and methods

Chemicals and reagents

All chemicals were of the highest purity and were purchased from Sigma–Aldrich (Tin Hang Technology Limited, Hong Kong) unless otherwise stated. Dynabeads (M-450 tosylactivated) and alkaline phosphatase-conjugated goat anti-human IgG, IgM and IgA antibodies were purchased from Life Technologies Limited (Hong Kong). Nanogold (10 nm) conjugated donkey anti-goat IgG was purchased from Abcam (Hong Kong) Limited. Restriction and ligation enzymes were purchased from New England Biolabs (Eastwin International Trading Limited, Hong Kong). HisTrap FF crude columns were purchased from GE Healthcare Bio-Sciences, Hong Kong. cOmplete Protease Inhibitor Cocktail was purchased from Roche Diagnostics, DKSH Hong Kong Limited, Hong Kong. RNeasy Mini Kits were purchased from Qiagen Hong Kong Pte, Hong Kong.

Assays

Anti-dsDNA Ab level was determined in serum samples from patients with lupus nephritis using Kallestad^{TM} anti-dsDNA microplate EIA (Bio-Rad Pacific Limited, Hong Kong). Lower and upper limits of detection were 20 and 300 IU/ml respectively, and values greater than 60 IU/ml were considered positive. Serum annexin II level was determined using a commercially available ELISA according to the manufacturer's instructions (LifeSpan BioSciences, Inc., Bio-Gene Technology Ltd., Hong Kong). Lower and upper limits of detection were 0.625 and 40 ng/ml respectively.

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Anti-dsDNA Ab levels were measured in sera obtained from NZBWF1/J mice using anti-dsDNA IgG quantitative ELISA kits according to the manufacturer's instructions (Alpha Diagnostic Inc., Onwon Trading Ltd., Hong Kong). Lower and upper limits of detection were 50 and 1000 IU/ml respectively, and values greater than mean + 2 standard deviation (S.D.) of sera from C57BL/6N mice were considered positive. Serum albumin, creatinine and urea levels were measured in control and NZBWF1/J mice using QuantiChrom™ Albumin, Creatinine and Urea Assay Kits respectively (BioAssay Systems, California, U.S.A.). Urine albumin-to-creatinine ratio was measured weekly in spot urine. Active nephritis in NZBWF1/J mice was confirmed by the presence of proteinuria >300 mg/dl on 2 separate occasions at least 2 days apart. All samples were measured in duplicate for all assays.

Patients and controls

The study included Chinese patients attending the SLE clinic at Queen Mary Hospital, Hong Kong, who had a history of biopsy-proven Class III/IV ± V lupus nephritis, with at least one episode of nephritic flare during the period of 2001–2013. Patients with serum samples obtained during active nephritis and remission were included for paired-sample analysis. Disease activity was classified as 'active' or 'inactive' based on clinical and serological parameters of disease. Active disease was confirmed by renal biopsy, significant proteinuria, active urinary sediment and an SLE disease activity index (SLEDAI) $\geqslant 10$, whereas inactive disease was defined as quiescent urinary sediment and an SLEDAI $\leqslant 4$ respectively [27]. Their active disease and remission serum samples were obtained at the time of kidney biopsy and during disease quiescence respectively. Patients with serial serum samples obtained at intervals of 3–4 months over a minimum of 24 months were selected for longitudinal studies. Patients with anti-phospholipid antibodies were excluded. All lupus nephritis patients received prednisolone with mycophenolate mofetil or cyclophosphamide as induction treatment during renal flare, followed by low-dose prednisolone and mycophenolate or azathioprine as long-term maintenance immunosuppression. Sex- and age-matched patients with non-lupus glomerular diseases (IgA nephropathy, diabetic nephropathy, minimal change disease, membranous nephropathy and ANCA-associated glomerulonephritis) (n=100) and healthy subjects (n=104) were included as renal disease controls and healthy controls respectively. Single serum samples were obtained from these subjects. The present study was approved by the Institutional Review Board of the University of Hong Kong/Hospital Authority Hong Kong West Cluster. All subjects gave written consent for the use of their serum and kidney tissue samples in the present study.

Serological and clinical parameters of disease

Serum IgG, IgA, IgM, C3 and creatinine levels at the corresponding serum sample time-points were retrieved from hospital records. Activity and chronicity indices were calculated based on renal histology parameters as previously described [28], and included assessment of glomerular cell proliferation, leucocyte infiltration, karyorrhexis/fibrinoid necrosis, hyaline deposits, cellular crescents and interstitial inflammation by an experienced renal pathologist. For each histology parameter, a scale of 0, 1, 2 or 3 + was used to indicate absent, mild, moderate and severe changes respectively. The activity score is the sum of individual scores of the aforementioned parameters, with the values of karyorrhexis/fibrinoid necrosis and cellular crescents multiplied by 2, and had a maximum value of 24 [28]. The chronicity index is the sum of individual scores for glomerular sclerosis, fibrous crescents, tubular atrophy and interstitial fibrosis, and scoring was as described above. The maximum value for chronicity index is 12 [28]. Electron-dense deposition in kidney tissues were also examined with transmission electron microscopy (TEM) and semi-quantitated using a scale of 0, 1, 2 or 3 + , indicating absent, mild, moderate or large amounts respectively.

Generation and purification of human and mouse annexin II

Recombinant human and mouse annexin II with 6× His-tag fused to the C-terminus was generated using the pET-21a bacterial expression system. Briefly, the full-length annexin II cDNA was amplified by PCR using human and mouse primers [for human annexin II: 5′-CGCATTAATACCATGTCTAC-3′ (forward) and 5′-CCGCTCGAGGGCGTCATCTCCACCACA-3′ (reverse); for mouse annexin II: 5′-CGCATTAATGCCACCATGTCTACTGTCCACGAA-3′ (forward) and 5′-GAAGTTATCAGTCGACGCCGTCATCCCCACCACACAGGT-3′ (reverse)], and digested with AseI and XhoI or SalI restriction enzymes. The purified insert was cloned into the bacterial expression vector pET-21a carrying a C-terminal His•Tag® sequence in frame between the NdeI and XhoI restriction sites. DNA plasmids were transformed into BL21(DE3)pLysS competent cells and annexin II protein expression induced by incubation with 0.1 mM IPTG at 37 °C for 3–4 h. Annexin II was purified from the cell lysate using HisTrap FF crude column, buffer exchanged into PBS and concentrated using Amicon Ultra-4 centrifugal filter devices. cOmplete Protease Inhibitor Cocktail was included in all buffers. The purity of human and mouse recombinant annexin II was >90%

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as confirmed by SDS/PAGE followed by Coomassie Blue staining. Recombinant annexin II was digested with DNase I to remove any residual DNA before use.

Measurement of serum immunoglobulin binding to annexin II in patients and mice with lupus nephritis

Serum Ig binding to annexin II was determined using an 'in-house' ELISA as previously described with slight modifications [6]. Briefly, for human serum samples, 96-well microtitre plates were coated overnight with DNA-free human recombinant annexin II (10 μg/ml) in 0.1 M carbonate buffer, pH 9.6. Plates were washed three times with PBS containing 0.05% Tween-20 (PBST) between incubations, and all incubations were for 1 h unless otherwise stated. Plates were blocked with 10% goat serum in PBST for 2 h at room temperature, and serum samples from lupus nephritis patients and controls (starting dilution 1:100) added in duplicate in serial dilution. Serum sample from a patient with active lupus nephritis that demonstrated high Ig binding to annexin II was used as the positive control and for inter-plate standardization, and was included in all plates. Alkaline phosphatase-conjugated goat anti-human IgG, IgM or IgA antibodies (1.4, 1.3 and 1.6 μg/ml respectively) were added, plates were developed with *p*-nitrophenyl phosphate and the absorbance was measured at 405 nm when the positive control showed an optical density (OD) of 1.5.

To determine annexin II-binding IgG and IgM levels in serum samples from NZBWF1/J and C57BL/6N mice, DNA-free mouse recombinant annexin II (5 μg/ml) was used to coat 96-well microtitre plates, samples (starting dilution 1:50) were added in duplicate in serial dilution, horseradish peroxidase-conjugated goat anti-mouse IgG or IgM antibodies (0.15 and 0.12 μg/ml respectively) were added, plates were developed with *O*-phenylenediamine dihydrochloride and the absorbance was measured at 450 nm. Seropositivity of serum IgG, IgM or IgA binding to annexin II was defined as samples having an OD that exceeded mean + 3 S.D. of samples from healthy subjects or C57BL/6N mice.

Animal studies

All animal procedures were approved by the Institutional Committee on the Use of Live Animals in Teaching and Research at the University of Hong Kong. Eight-week-old female NZBWF1/J mice were purchased from Jackson Laboratory (Bar Harbor, U.S.A.) and C57BL/6N mice were purchased from Laboratory Animal Unit at the University of Hong Kong. Mice were housed in a pathogen-free animal facility and kept under normal housing conditions in a 12-hour night and day cycle. Water and standard chow were available ad libitum. Mice were randomized into seven age groups, which represented pre-nephritic mice (14 weeks old), progressive proliferative disease (20, 24, 28 and 32 weeks old) and severe disease with glomerulosclerosis (36 and 40 weeks old) (n=6 per group per time-point). At the aforementioned time-points, mice were killed, sera collected and kidneys harvested for histological assessment.

Isolation of glomeruli from control and NZBWF1/J mice

Glomeruli were isolated from the kidneys of control and NZBWF1/J mice according to the method of Takemoto et al. [29], with slight modifications. Briefly, mice were anaesthetized by intraperitoneal injection of midazolam (6.25 mg/kg body weight) and fentanyl citrate (3.33 mg/kg body weight) and 8×10^7 spherical iron-containing Dynabeads in 40 ml Hank's balanced salt solution (HBSS) administered by intracardiac perfusion using a peristaltic pump. Unless otherwise stated, all subsequent procedures were undertaken at 4°C. Kidneys were harvested, cut into small pieces and digested with collagenase (1 mg/ml) and DNase I (100 U/ml) in HBSS at 37°C for 30 min with gentle agitation before gently passing through 100 μm cell strainers. Samples were washed with HBSS, the filtrates passed through new cell strainers and the contents centrifuged at 200 × **g** for 5 min. Supernatants were discarded, cell pellets resuspended in HBSS (2 ml) and glomeruli containing Dynabeads concentrated on a magnetic stand.

Gene expression of annexin II and p11 using quantitative real-time PCR

Isolated glomeruli were washed five times with HBSS and total mRNA extracted using RNeasy mini kits according to the manufacturer's instructions. One microgram of total RNA was reverse-transcribed into cDNA with M-MLV reverse transcriptase using the random hexamer method [6]. Annexin II and p11 gene expression was assessed by quantitative real-time PCR using Taqman gene expression assays (gene expression assay no. Mm01150673_m1 and Mm00501457_m1 respectively) on a Lightcycler 480 II real-time PCR system (Roche Diagnostics, DKSH Hong Kong Limited, Hong Kong). All samples were analysed in triplicate, and annexin II and p11 mRNA expression calculated using the $\Delta\Delta C_t$ ($2^{-\Delta\Delta C_t}$) method, normalized to GAPDH.

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Expression of annexin II and p11 in kidneys of control and NZBWF1/J mice

Paraffin-embedded kidney sections (5 μm) from C57BL/6N and NZBWF1/J mice at different stages of disease were stained for annexin II and p11 as previously described [30,31]. Signal detection and visualization was by the peroxidase-anti-peroxidase method and counterstaining was with haematoxylin. Intra-glomerular staining of annexin II and p11 was semi-quantitatively assessed in a blinded manner in approximately 20 glomeruli per mouse kidney using a Nikon Eclipse Ni-U upright microscope [Chinetek Scientific (China) Limited, Hong Kong] and graded as follows: 0 = 0–5% staining, 1 = >5–25% staining, 2 = >25–50% staining, 3 = >50–75% staining, 4 = >75% staining. The reproducibility of this scoring system has been documented [32].

Assessment of annexin II and p11 co-localization by immunofluorescence

Annexin II and p11 co-localization in the kidneys of control and NZBWF1/J mice was assessed by indirect immunofluorescence using snap frozen renal specimens (8 μm) as previously described [6]. Sections were mounted in fluorescent mountant and epifluorescence viewed using a Nikon Eclipse Ni-U upright fluorescent microscope. Images were captured using a Spot RT3 cooled CCD camera and Spot 5.0 Software (Diagnostic Instrument Inc., Michigan, U.S.A.).

TEM and immunogold staining

Kidney specimens for TEM and immunogold staining were prepared as previously described with slight modifications [33]. Briefly, ultrathin sections (100 nm) of paraformaldehyde–glutaraldehyde fixed kidneys, post-fixed with 0.1% osmium tetroxide were embedded in epoxy resin and mounted onto copper grids. Sections were stained with 2% aqueous uranyl acetate and Reynold's lead citrate, and examined by TEM using an EM208s microscope (Philips Eindhoven).

For immunogold staining, kidneys were fixed with paraformaldehyde–glutaraldehyde, embedded in LR white resin (medium grade) and ultrathin sections mounted onto nickel grids. Sections were washed with 0.05 M glycine in PBS and blocked with AURION donkey serum blocking solution. Sections were then washed twice with PBS containing BSA and sodium azide (PBS–BSA–NaN_3 buffer) and incubated overnight with goat anti-annexin II Ab (diluted 1:20 in PBS–BSA–NaN_3 buffer) at 4 °C, after which time sections were washed with PBS–BSA–NaN_3 buffer and incubated with 10 nm gold-labelled donkey anti-goat IgG secondary Ab. Sections were counterstained with uranyl acetate and lead citrate and examined by TEM.

Statistical analyses

Results are expressed as mean $\pm$ S.D. Statistical analyses were performed using GraphPad Prism 5.01 for Windows (La Jolla, California, U.S.A.). Analysis of covariance with Bonferroni's multiple comparison post-test or Kruskal–Wallis with Dunn's multiple comparison test was used to compare differences between groups, as appropriate. Fisher's exact test was used to compare serum annexin II-binding Ig seropositivity between groups. Correlation between serum Ig binding to annexin II and serological and clinical parameters of disease was assessed by the Spearman's rank correlation coefficient. Two-tailed $P < 0.05$ was considered statistically significant.

Results

Patient characteristics

Forty-five lupus nephritis patients (34 female and 11 male) were included for paired-sample analysis (Table 1). Their serum samples included one sample obtained during active disease and another sample during remission. Twenty-eight lupus nephritis patients (18 females and 10 males) were included for longitudinal studies, with serial serum samples obtained at intervals of 3–4 months over a follow-up period of 6.4 ± 2.8 years. Of the total serum samples used in the present study (n=563), less than 5% (9 and 13 serum samples from lupus nephritis patients with active disease and in remission respectively) were also included in our previous study [6].

Annexin II-binding immunoglobulins in serum

The mean levels of serum annexin II-binding IgG in healthy subjects, patients with non-lupus renal diseases, lupus nephritis patients in remission and lupus nephritis patients during flare were 0.36 ± 0.17, 0.31 ± 0.18, 0.44 ± 0.45 and 0.39 ± 0.29 OD respectively, which were similar in the four groups (Figure 1A). In contrast, seropositivity rates for

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Table 1 Characteristics of 45 patients with lupus nephritis

	Active disease	Remission	*P* value
Female: Male	34: 11		–
Age (years)	41.1 ± 11.8		–
Anti-dsDNA antibodies (IU/ml)	433.9 ± 485	107.1 ± 129.5	<0.0003
IgG (mg/dl)	1127 ± 466	1161 ± 557.5	0.7648
IgM (mg/dl)	87.9 ± 76.59	70.3 ± 50.3	<0.0074
IgA (mg/dl)	231.6 ± 145.1	218.1 ± 154.2	0.1088
Creatinine (μM/l)	117.5 ± 78.7	109.0 ± 77.9	0.1577
C3 (mg/dl)	48.0 ± 15.27	80.0 ± 25.47	<0.0001

annexin II-binding IgG were 0.9%, 1.3%, 15.6% and 8.9% in the four groups respectively ($P < 0.01$, active lupus nephritis or remission lupus nephritis compared with healthy subjects; $P = 0.004$, remission lupus nephritis compared with non-lupus renal diseases; $P =$ NS (not significant), active lupus nephritis compared with remission lupus nephritis and non-lupus renal diseases compared with healthy subjects). Annexin II-binding IgM level was 0.60 ± 0.41, 0.64 ± 0.46, 0.44 ± 0.45 and 1.01 ± 1.17 OD in the four groups, showing a significantly higher level in patients with active lupus nephritis (Figure 1B and 1E), with seropositivity rates of 1.9%, 4.0%, 2.2%, and 11.1% respectively ($P = 0.02$, active lupus nephritis compared with healthy subjects; $P =$ NS, active lupus nephritis compared with remission lupus nephritis, active or remission lupus nephritis compared with non-lupus renal diseases and healthy subjects compared with non-lupus renal diseases). Annexin II-binding IgA level was 0.35 ± 0.37, 0.36 ± 0.39, 0.44 ± 0.45 and 0.36 ± 0.42 OD respectively (Figure 1C and 1F), with seropositivity rates of 2.9%, 3.0%, 4.4% and 4.4% respectively. Since there was no significant between-group difference for both the mean value and seropositivity rates for serum IgA binding to annexin II, further investigations were performed for IgG and IgM only.

Annexin II-binding IgG/IgM and lupus nephritis disease parameters

While the mean level of annexin II-binding IgG in serum did not differ among lupus nephritis patients during flare or remission (Figure 1D), the level of annexin II-binding IgM was significantly higher during active lupus nephritis ($P = 0.0029$) (Figure 1E). Annexin II-binding IgG level, but not that of IgM correlated with the level of anti-dsDNA Ab (r=0.249, $P = 0.027$) (Figure 2A and 2B). Annexin II-binding IgG and IgM inversely correlated with serum C3 level ($r = -0.239$, $P = 0.036$ and $r = -0.377$, $P = 0.0006$ respectively) (Figure 2C and 2D).

Relationship between the level of annexin II-binding IgG or IgM, anti-dsDNA level and disease activity in longitudinal studies

Of the 28 patients included, 12 (42.9%) showed a temporal relationship between serum annexin II-binding IgG, anti-dsDNA level and disease activity, while 15 patients (53.6%) showed a temporal relationship between annexin II-binding IgM, anti-dsDNA level and disease activity, i.e. high levels during active disease that decrease as the disease went into remission after treatment as demonstrated by illustrative patients (Figure 3). In 6 patients (21.4%), both annexin II-binding IgG and IgM levels were associated with disease activity and anti-dsDNA level.

Annexin II-binding IgG/IgM and kidney histopathology

Kidney biopsies from 22 lupus nephritis patients, all obtained at diagnosis of active nephritis prior to potent immunosuppressive therapy, were studied. The level of annexin II-binding IgG correlated with lupus nephritis activity index (r=0.441, $P = 0.040$), individual histologic lesions including glomerular leucocyte infiltration score (r=0.520, $P = 0.018$), karyorrhexis/fibrinoid necrosis score (r=0.657, $P < 0.002$) and the quantity of mesangial electron-dense deposits (r=0.630, $P = 0.009$) (Figure 4A–D). Annexin II-binding IgM level correlated with overall activity index (r=0.430, $P < 0.045$) (Figure 5), but not with individual parameters associated with histologic activity. Annexin II-binding IgG and IgM levels were not associated with the chronicity index (results not shown).

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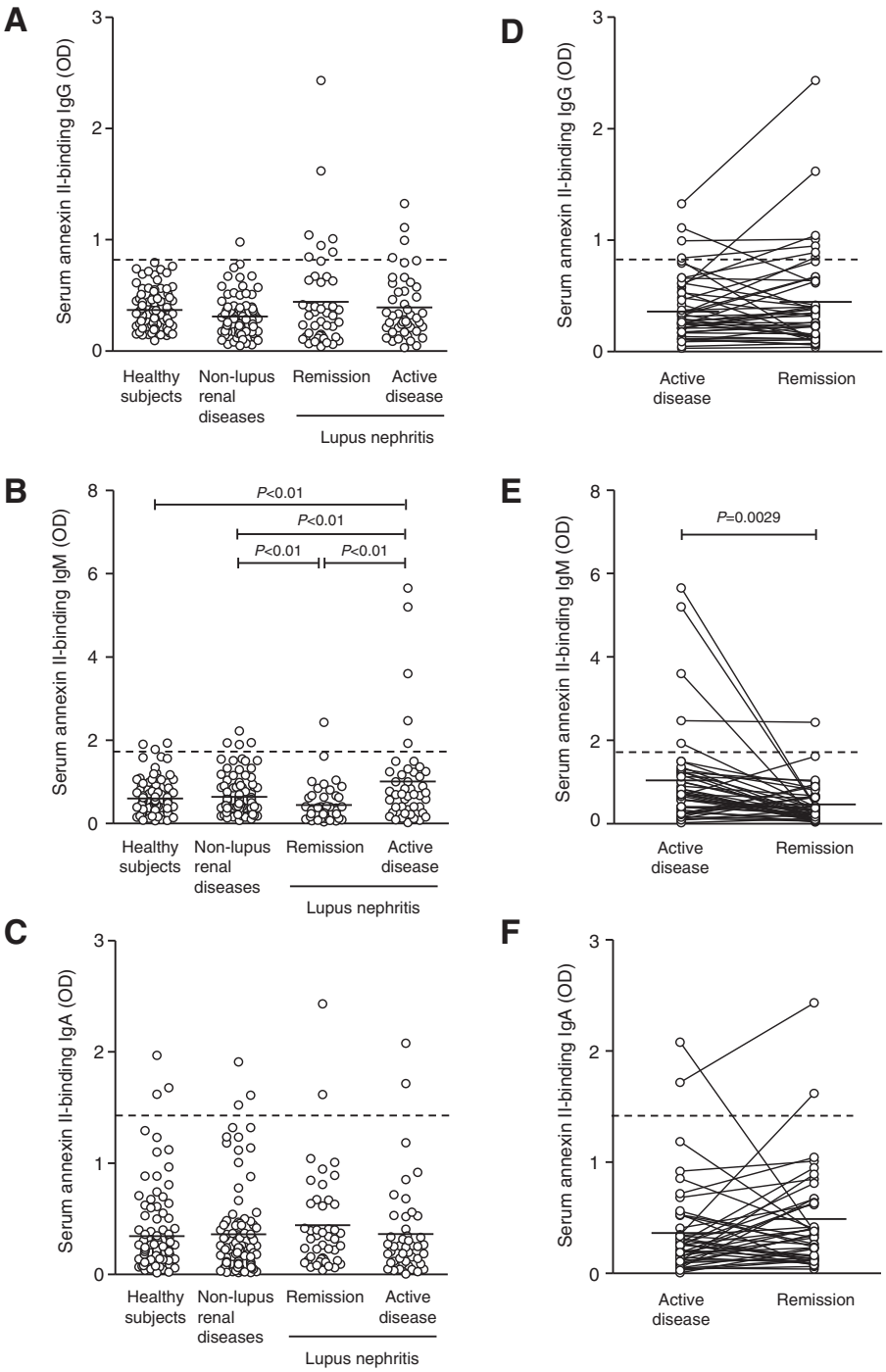

Figure 1. Annexin II-binding Igs in serum of healthy subjects, patients with non-lupus renal diseases and patients with lupus nephritis

Scatterplots showing serum (**A**) IgG, (**B**) IgM and (**C**) IgA binding to annexin II in healthy subjects, patients with non-lupus renal diseases, lupus nephritis patients in remission and lupus nephritis during active disease. Comparison of annexin II-binding (**D**) IgG, (**E**) IgM and (**F**) IgA in paired serum samples from 45 lupus nephritis patients during active disease and remission. Dashed line represents mean OD of healthy subjects + 3 S.D., and values above this line are seropositive. Horizontal line represents mean for each group.

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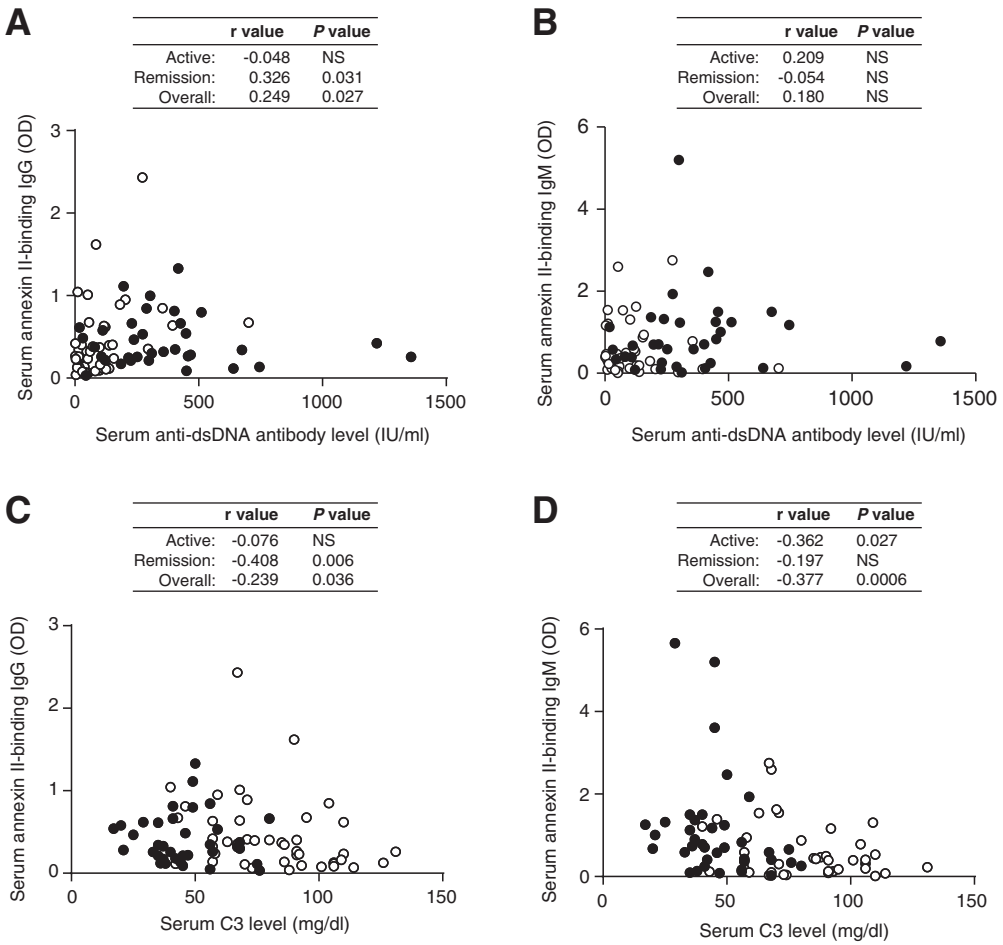

Figure 2. Relationship between annexin II-binding Ig level with anti-dsDNA Ab and C3 levels

(**A**) Serum annexin II-binding IgG level, but not (**B**) serum annexin II-binding IgM level, correlated with anti-dsDNA Ab level. Serum (**C**) annexin II-binding IgG level and (**D**) annexin II-binding IgM level inversely correlated with C3 level. Black and white circles represent annexin II-binding IgG or IgM levels in patients during active disease and remission respectively.

Serum annexin II level in lupus nephritis patients

The level of annexin II in serum was 0.20 ± 0.10, 0.31 ± 0.34, 0.40 ± 0.19 and 0.48 ± 0.35 ng/ml in healthy subjects, patients with non-lupus renal diseases, lupus nephritis patients in remission and lupus nephritis patients during flare respectively ($P < 0.001$, active lupus nephritis compared with healthy subjects; $P < 0.05$, active lupus nephritis compared with non-lupus renal diseases, $P =$ NS, active lupus nephritis compared with remission lupus nephritis (Figure 6). Seropositivity rates were 1.7%, 9.5%, 30.3% and 36.4% for healthy subjects, patients with non-lupus renal diseases, lupus nephritis patients in remission and patients with active lupus nephritis respectively ($P < 0.0001$, active or remission lupus nephritis compared with healthy subject; $P < 0.01$, active or remission lupus nephritis compared with non-lupus renal diseases, $P =$ NS, active lupus nephritis compared with remission lupus nephritis and healthy subjects compared with non-lupus renal diseases). Serum annexin II level in lupus nephritis patients was not related to that of annexin II-binding IgG/IgM, anti-dsDNA Ab level or disease activity (results not shown).

Annexin II, p11 and serum annexin II-binding IgG and IgM in NZBWF1/J mice

Anti-dsDNA antibodies were produced by NZBWF1/J mice at 14 weeks of age, before the onset of proteinuria. Anti-dsDNA Ab level peaked at 28 weeks of age and remained elevated thereafter. Increased urine albumin-to-creatinine ratio and serum urea level became evident at 24–28 weeks of age, with further

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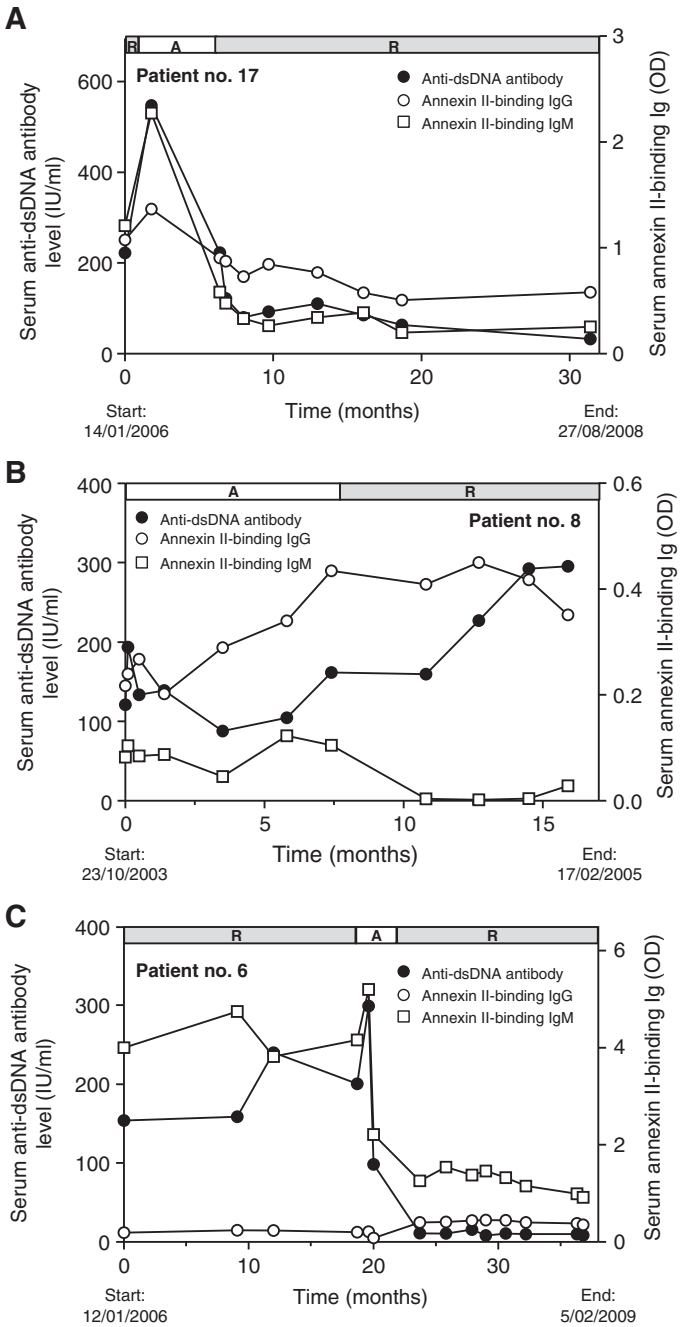

Figure 3. Serial levels of annexin II-binding IgG/IgM and anti-dsDNA in relation to disease activity in patients with lupus nephritis

Representative graphs from three lupus nephritis patients showing the association between serial annexin II-binding IgG or IgM profile and the level of anti-dsDNA in relation to disease activity. The patient represented by panel (**A**) showed high levels of annexin II-binding IgG and IgM as well as anti-dsDNA during active disease, which decreased as the nephritis went into remission after treatment. Panel (**B**) represents a patient who showed high levels of annexin II-binding IgG, but not IgM, and anti-dsDNA during active nephritis. Panel (**C**) represents a patient who showed high levels of annexin II-binding IgM, but not IgG, and anti-dsDNA during active nephritis. A = active nephritis, R = remission

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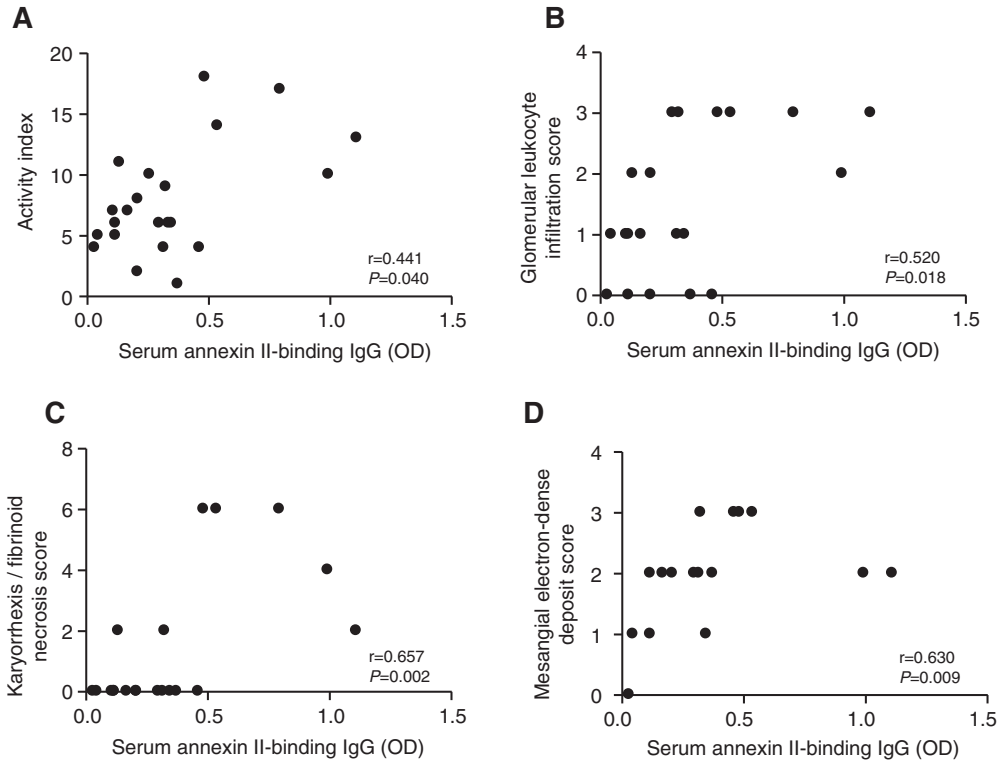

Figure 4. Annexin II-binding IgG level in serum and renal histology in kidney biopsies of patients with lupus nephritis

Serum annexin II-binding IgG level correlated with (**A**) activity index, (**B**) glomerular leucocyte infiltration score, (**C**) karyorrhexis/fibrinoid necrosis score and (**D**) mesangial electron-dense deposit quantitation score in kidney biopsies showing active lupus nephritis.

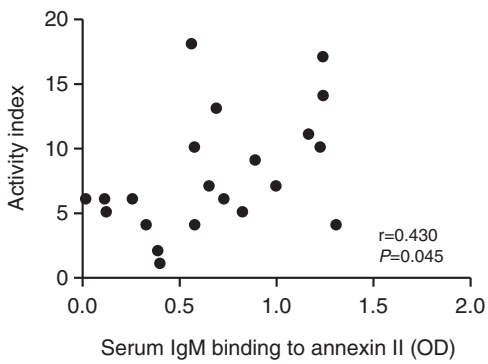

Figure 5. Annexin II-binding IgM level in serum and renal histology in kidney biopsies of patients with lupus nephritis

Serum annexin II-binding IgM level correlated with activity index.

deterioration as disease progressed (Figure 7). Mean serum annexin II-binding IgG and IgM levels were 0.016 ± 0.025 and 0.035 ± 0.027 OD respectively in C57BL/6N mice at 14 weeks of age and the levels did not change with age. None of the control mice were seropositive for annexin II-binding IgG or IgM. Mean serum annexin II-binding IgG level in NZBWF1/J mice at 14, 24 and 40 weeks of age were 0.036 ± 0.020, 0.055 ± 0.017 and 0.081 ± 0.039 OD respectively ($P < 0.01$, C57BL/6N compared with NZBWF1/J mice at 40 weeks of age) (Figure 8A) and seropositivity rates were 0%, 16.7% and 83.3% respectively ($P < 0.01$, C57BL/6N compared with NZBWF1/J mice at 40 weeks of age, $P =$ NS, C57BL/6N compared with NZBWF1/J mice at 14 and 24 weeks of age). Mean serum annexin II-binding IgM level in NZBWF1/J mice at 14, 24 and 40 weeks of age was 0.056 ± 0.041, 0.055 ± 0.026 and 0.134 ± 0.117 OD respectively ($P < 0.01$, C57BL/6N compared with NZBWF1/J mice at 40 weeks of age) (Figure 8B), with seropositivity rates of

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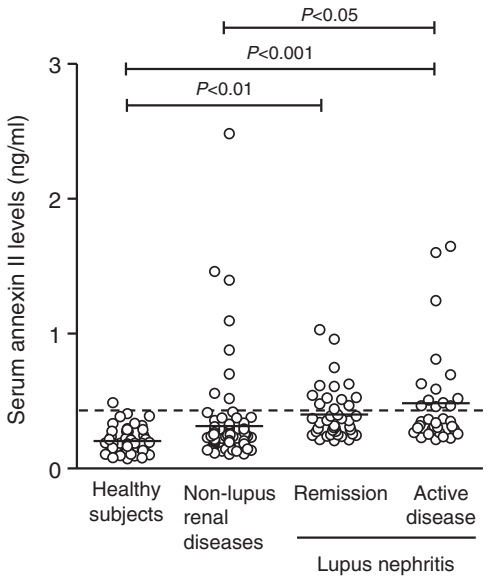

Figure 6. Serum annexin II level in healthy subjects, patients with non-lupus renal diseases and patients with lupus nephritis

Scatterplots showing serum annexin II levels in healthy subjects, patients with non-lupus renal diseases, lupus nephritis patients in remission and lupus nephritis during active disease. Dashed line represents mean OD of healthy subjects + 3 S.D., and values above this line are seropositive. Horizontal line represents mean for each group.

16.67%, 16.67% and 66.7% respectively. Although four out of six NZBWF1/J mice showed seropositivity for annexin II-binding IgM at 40 weeks of age, it did not reach statistical significance ($P = 0.06$) ($P =$ NS, C57BL/6N compared with NZBWF1/J mice at 14 and 24 weeks of age).

Glomeruli were isolated from control and NZBWF1/J mice using Dynabeads, and light microscopy was performed to confirm that there was no contamination with non-glomerular tissue (Supplementary Figure S1A and S1B). TEM confirmed the entrapment of Dynabeads in the glomerular capillaries (Supplementary Figure S1C). Annexin II and p11 gene and protein expression were similar between pre-nephritic NZBWF1/J mice and age-matched control mice (Figures 9 and 10). Annexin II and p11 expression did not increase with age in control mice. Gene and protein expression of annexin II and p11 increased in NZBWF1/J mice from 24 to 32 weeks of age when their kidneys showed progressive nephritis, and decreased afterwards in areas where cell proliferation was reduced and fibrotic lesions apparent (Figures 9 and 10). At 36 and 40 weeks of age, $35.7 \pm 14.4\%$ and $65.9 \pm 20.0\%$ of glomeruli respectively in NZBWF1/J mice showed global sclerosis. Annexin II co-localized with p11 in the glomerulus (Figure 11). At the ultrastructural level, immunogold labelling of annexin II was rarely detected in mesangial cells and endothelial cells in control mice (Figure 12A). In NZBWF1/J mice, increased immunogold labelling of annexin II was observed along the cell membrane of mesangial cells, within the mesangial matrix (MM) and in electron-dense immune deposits present in the GBM (Figure 12B).

Discussion

Annexin II autoantibodies have been detected in patients with rheumatoid arthritis, APS, SLE and lung cancer [25,34]. We have previously reported that polyclonal IgG anti-dsDNA antibodies isolated from patients with diffuse proliferative lupus nephritis can bind annexin II, and the degree of binding correlated with circulating anti-dsDNA Ab level [6]. Subpopulations of anti-dsDNA antibodies with annexin II cross-reactivity therefore contribute to the repertoire of annexin II-binding Ig [6]. The relationship between annexin II-binding Ig and serological, clinical or histological parameters of disease has not been investigated.

In the present study, we demonstrated that seropositivity rates for annexin II-binding IgG and IgM, but not IgA, were significantly higher in patients with Class III/IV ± V lupus nephritis compared with healthy subjects and patients with non-lupus renal diseases. Similar findings were also noted in NZBWF1/J mice when compared with control mice. Although the mean level of annexin II-binding IgG did not differ among lupus nephritis patients during active

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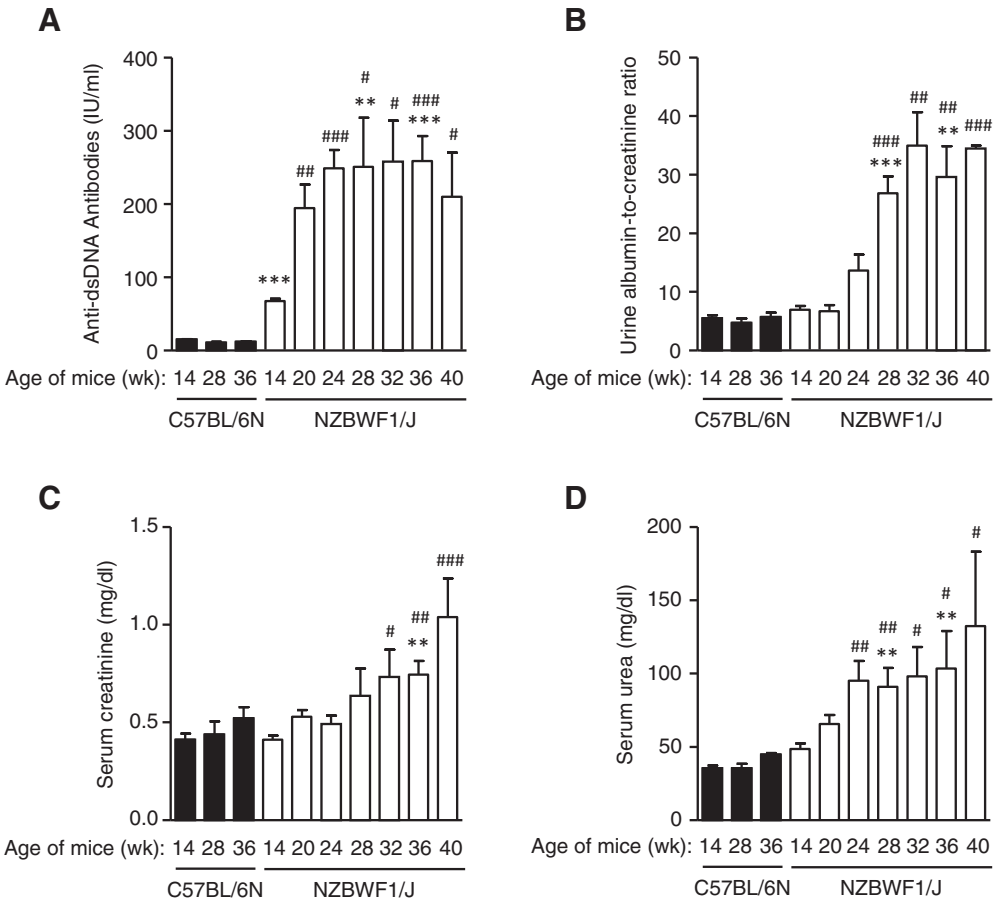

Figure 7. Urine albumin-to-creatinine ratio and serum anti-dsDNA, creatinine and urea levels in control and NZBWF1/J mice

(**A**) Serum anti-dsDNA Ab, (**B**) urine albumin-to-creatinine ratio, (**C**) serum creatinine and (**D**) serum urea levels were determined in C57BL/6N and NZBWF1/J mice using commercially available assays. Data are presented as mean ± S.D. with six mice per time-point per group; $^{**}P < 0.01$, $^{***}P < 0.001$, control compared with NZBWF1/J mice of the same age; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$, compared with NZBWF1/J mice at 14 weeks of age.

disease and those in remission, annexin II-binding IgM was significantly higher in lupus nephritis patients during nephritic flare. Both annexin II-binding IgG and IgM showed temporal relationships with disease activity, and serum anti-dsDNA Ab and C3 levels suggesting their putative roles in the pathogenesis of lupus nephritis. Furthermore, annexin II-binding IgG and IgM levels correlated with histological activity index but not chronicity index, suggesting that these Igs may contribute to acute injury in active lupus nephritis. The seropositivity rate for annexin II level was significantly higher in lupus nephritis patients compared with control groups, and showed no statistical difference between active disease and remission. Serum annexin II level showed no association with annexin II-binding IgG or IgM levels, or clinical, serological or histological parameters of disease.

Studies on annexin II-binding Igs in SLE patients are limited [14,26], and have focused mainly on those belonging to the IgG subclass. In this respect, IgM anti-ssDNA antibodies form the normal repertoire of natural antibodies in healthy subjects, but can undergo IgG switch in lupus patients, which increases their pathogenic potential [35]. IgG anti-dsDNA Ab level often correlates with disease activity in lupus nephritis patients [4,35,36], whereas serum IgM anti-dsDNA Ab level has been reported to show a negative association with glomerulonephritis and is thought to be non-pathogenic [37]. It is of interest to note the association between annexin II-binding IgM and activity index in the renal biopsy samples, and its inverse correlation with C3 level, while there was no association with anti-dsDNA Ab level. These results suggest both a pathogenic role and characteristics of IgM anti-annexin II that are distinct from anti-dsDNA antibodies. Whether IgM anti-dsDNA antibodies can cross-react with annexin II warrants further investigation. Of the numerous pro-inflammatory cytokines synthesized in lupus patients, interferon has been

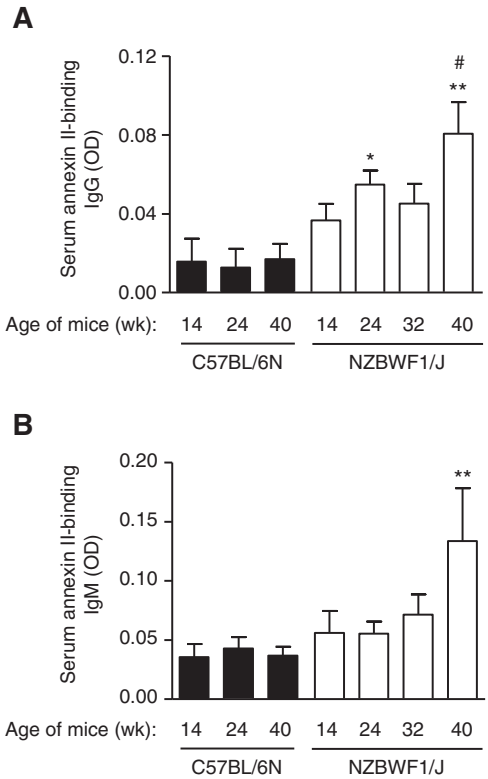

Figure 8. Annexin II-binding Igs in serum of control and NZBWF1/J mice

Bar charts showing serum annexin II-binding (**A**) IgG and (**B**) IgM in C57BL/6N and NZBWF1/J mice. The age groups of NZBWF1/J mice represent different stages of disease where 14 weeks of age represents pre-nephritic stage, 24 and 32 weeks of age represents proliferative nephritis, and 40 weeks of age represents severe disease with glomerulosclerosis. Data are presented as mean ± S.D. with six mice per time-point per group; $^{*}P < 0.05$, $^{**}P < 0.01$, control compared with NZBWF1/J mice of the same age; $^{\#}P < 0.05$, compared with NZBWF1/J mice at 14 weeks of age.

implicated in both systemic and end-organ inflammation [38]. Recently, it has been reported that induction of the interferon signature required opsonization of apoptotic cells by C3 and IgM in pristane-induced lupus mice [39]. In line with our study, this would suggest that subsets of IgM antibodies possess pathogenic potential in lupus.

Seropositivity rates for annexin II-binding IgG in the present study were 8.9% and 15.6% in lupus nephritis patients with active disease and remission respectively. In SLE patients without APS or thrombosis, seropositivity rates for annexin II-binding IgG have been reported to range from 3.4% to 7.5% compared with 2.1% in healthy subjects [25,26]. In a separate study, Caster et al. [14] reported that serum samples from ten patients with proliferative lupus nephritis all demonstrated seropositivity for annexin II-binding IgG, whereas there was no apparent difference between annexin II-binding IgG level in patients with membranous lupus nephritis, SLE patients without nephritis or healthy subjects. All patients recruited in our study had Class III/IV ± V lupus nephritis and none were solely Class V. Although our seropositivity rates are in line with those reported in SLE patients without APS or thrombosis, no information is available on whether these patients had nephritis or otherwise. The discrepancy in seropositivity rates for annexin II-binding IgG between our study and that of Caster et al. [14] may be related to the differences in sample size and patient characteristics. The small percentage of lupus nephritis patients who were seropositive for annexin II-binding IgG and IgM may suggest that only subpopulations of lupus patients have elevated circulating autoantibodies to annexin II during disease activity. Alternatively, the interaction of annexin II-binding Igs with annexin II in the bloodstream or their deposition in the kidney may also contribute to the low seropositivity rates although further studies are warranted to confirm this.

Activity and chronicity indices have been devised to quantitate acute injury and chronic damage respectively in the glomerular, tubulo-interstitial and vascular compartments of the kidney [28,40]. In the present study, we demonstrated that annexin II-binding IgG level in lupus nephritis patients correlated with the histological activity index,

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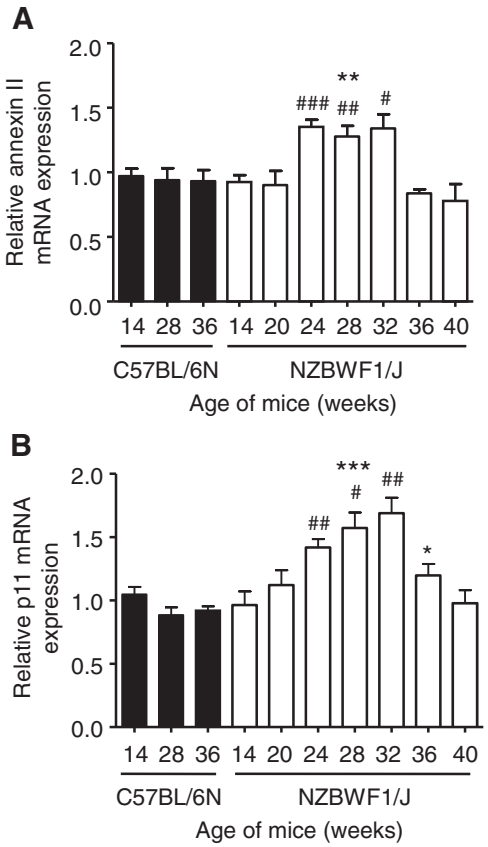

Figure 9. Annexin II and p11 gene expression in the glomeruli of C57BL/6N and NZBWF1/J mice

Gene expression of (**A**) annexin II and (**B**) p11 in glomeruli of C57BL/6N and NZBWF1/J mice were determined by real-time PCR, and values normalized to the housekeeping gene GAPDH. The age groups of NZBWF1/J mice represent different stages of disease where 14 weeks of age represents pre-nephritic stage, 24 to 32 weeks of age represents proliferative nephritis, and 36 and 40 weeks of age represents severe disease with glomerulosclerosis. Data are presented as mean ± S.D. relative to the level detected in control mice at 14 weeks of age; $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, control compared with NZBWF1/J mice of the same age; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$, compared with NZBWF1/J mice at 14 weeks of age.

especially leucocyte infiltration and karyorrhexis/fibrinoid necrosis, and the amount of mesangial electron-dense deposits. Annexin II-binding IgM level correlated with activity index, further suggesting that this subgroup of IgM autoantibodies are pathogenic, and together with annexin II-binding IgG may contribute to acute glomerular injury in lupus nephritis patients. Macrophages play important roles in the pathogenesis of lupus nephritis since their depletion in an inducible murine model of lupus nephritis using a selective CSF-1R kinase inhibitor preserved kidney histology and function, and improved proteinuria [41]. Monocytes and macrophages constitutively express cell surface annexin II. Also, increased annexin II expression is observed in activated peritoneal macrophages, which regulate plasminogen-dependent macrophage/monocyte recruitment to sites of injury [42]. Whether the increased annexin II expression in active lupus nephritis has implications for pathogenic processes, which involve macrophages, remains speculative.

Karyorrhexis, neutrophil exudation, basement membrane fragmentation, fibrin deposition and fibrinoid necrosis of the glomerular tuft are lesions signifying activity in proliferative lupus nephritis [43]. Annexin II possesses anti-fibrinolytic activity through its ability to inhibit plasmin-dependent fibrinolysis [44,45]. In addition to annexin II serving as a cross-reactive antigen that mediates the binding of anti-dsDNA antibodies to mesangial cells thereby initiating downstream inflammatory processes, it is possible that annexin II may also be involved in the pro-fibrotic or reparative processes in the glomerular lesions of lupus nephritis.

We have previously demonstrated that annexin II co-localizes with intra-glomerular IgG and C3 deposits in human and murine lupus nephritis [6]. The amount of immune complexes deposited in the kidney and their location

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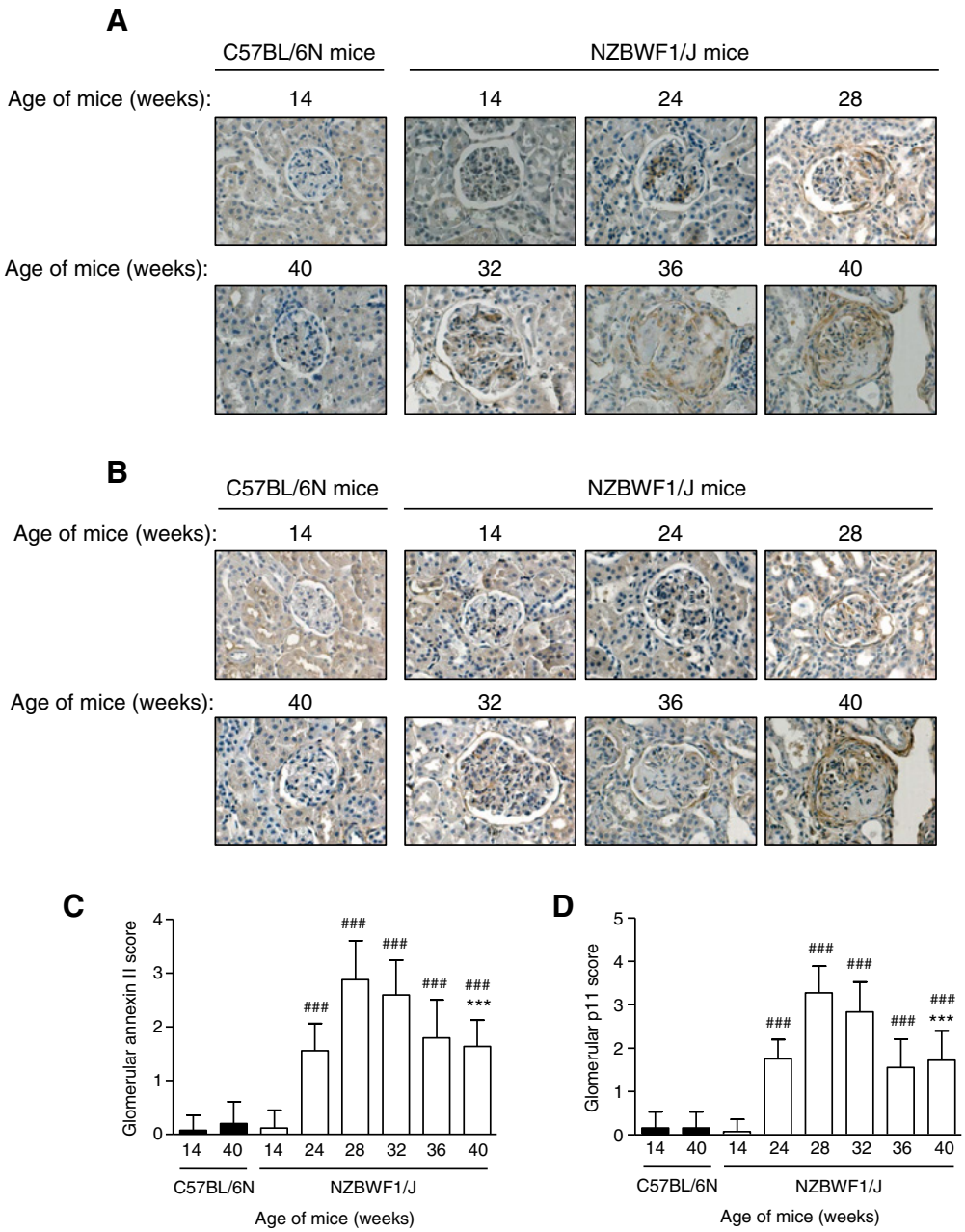

Figure 10. Annexin II and p11 protein expression in the glomeruli of C57BL/6N and NZBWF1/J mice

Representative images showing glomerular expression of (**A**) annexin II and (**B**) p11 in control and NZBWF1/J mice. The age groups of NZBWF1/J mice represent different stages of disease where 14 weeks of age represents pre-nephritic mice, 24 to 32 weeks of age represents proliferative nephritis, and 36 and 40 weeks of age represents severe disease with glomerulosclerosis. Original magnification ×600. Glomerular expression of (**C**) annexin II and (**D**) p11 were graded as described in the methodology and results expressed as mean ± S.D; $^{***}P < 0.001$, control compared with NZBWF1/J mice of the same age, $^{###}P < 0.001$, compared to NZBWF1/J mice at 14 weeks of age.

correlate with the severity of pathogenesis [46]. The exact location of annexin II within the glomerulus and changes in its expression during progressive lupus nephritis remain to be defined. In control mice, annexin II and its binding protein p11 were weakly expressed in the glomerulus and their expression did not alter with age. Intra-glomerular expression of annexin II and p11 was markedly increased in NZBWF1/J mice with active nephritis especially in glomeruli showing proliferative features. Both annexin II and p11 expression were absent in areas of fibrosis and decreased cell number. At the ultrastructural level, immunogold-labelled annexin II was only rarely observed in mesangial cells

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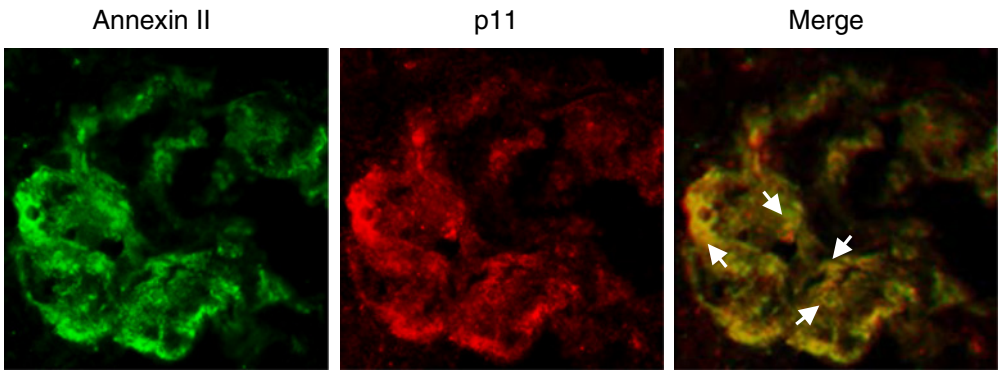

Figure 11. Co-localization of annexin II and p11 in NZBWF1/J mice glomeruli

Representative images showing intra-glomerular annexin II (green) and p11 expression (red) in NZBWF1/J mice at 32 weeks of age. Co-localization of annexin II and p11 within the glomerulus is depicted by yellow colouration (depicted by arrows) in the image labelled 'Merge'. Original magnification ×400.

of control mice. In contrast, annexin II expression was markedly increased in NZBWF1/J mice with active disease and was detected along the plasma membrane of mesangial cells, within the MM and GBM, and in electron-dense deposits present along the GBM. Their presence in mesangial deposits as well as the GBM suggest a pathological role in lupus nephritis. Immunogold-labelled annexin II was also detected in podocytes and endothelial cells in NZBWF1/J mice but due to their dark cytoplasmic content, further studies are necessary to confirm this.

In conclusion, we demonstrated increased seropositivity rates for annexin II-binding IgG and IgM in patients and mice with lupus nephritis. Annexin II-binding IgG and IgM levels were associated with anti-dsDNA Ab level, disease activity, histological activity and quantity of mesangial electron-dense deposits. Increased annexin II expression was observed in NZBWF1/J mice with proliferative nephritis, and was predominantly localized to mesangial cells, MM and in electron-dense deposits in the GBM. That annexin II and Igs that bind to annexin II are involved in the pathogenesis of active lesions in lupus nephritis is further corroborated by the finding of decreased annexin II expression in areas of fibrosis.

Clinical perspectives

- Lupus nephritis is a severe manifestation of SLE and an important cause of kidney failure in many parts of the world. Annexin II mediates anti-dsDNA Ab binding to mesangial cells and triggers downstream inflammatory processes in the renal parenchyma. The clinical associations and pathogenic significance of annexin II-binding Igs in lupus nephritis have not been investigated.

- Seropositivity rates for annexin II-binding IgG and IgM were significantly higher in lupus nephritis patients compared with healthy subjects and patients with non-lupus renal diseases. Annexin II-binding IgM level was increased during active disease, and annexin II-binding IgG and IgM levels correlated with the level of anti-dsDNA Ab and disease activity in 42.9% and 53.6% of lupus nephritis patients respectively. Annexin II-binding IgG and IgM levels correlated with histological activity index and its constituent parameters in kidney biopsies, and also the amount of electron-dense deposits in the glomerular mesangium. Annexin II and p11 expression was increased in glomeruli showing proliferative lupus nephritis. At the ultrastructural level, annexin II localized to the surface of mesangial cells and in the MM, and also with electron-dense immune deposits along the GBM.

- Our results suggest that annexin II and Igs that bind annexin II are associated with clinical and histological disease activity in the pathogenesis of lupus nephritis.

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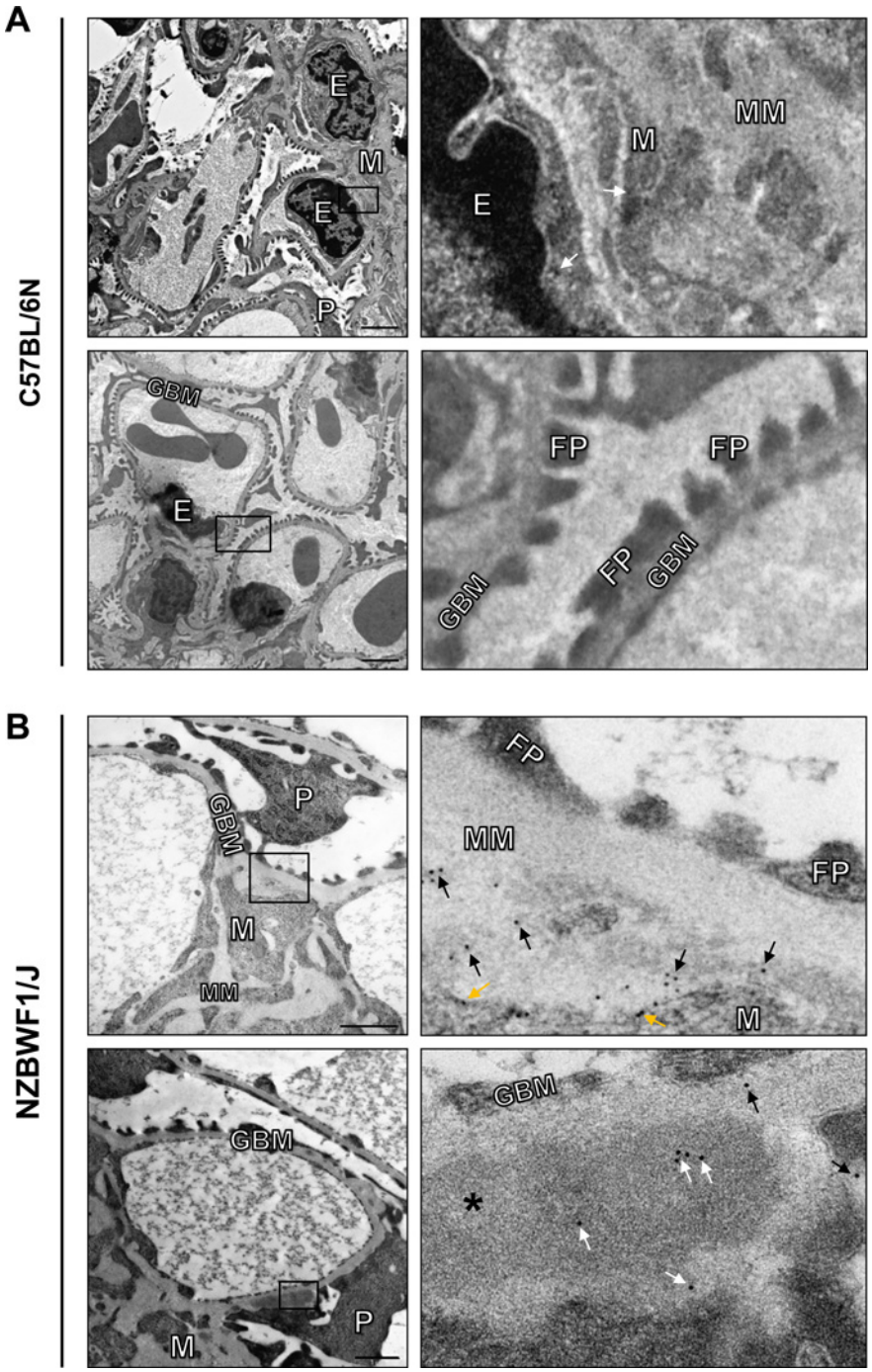

Figure 12. Ultrastructural localization of annexin II in the glomeruli of control and NZBWF1/J mice with immunogold staining and TEM

Representative images showing ultrastructural localization of immunogold-labelled annexin II in the kidneys of (**A**) control and (**B**) NZBWF1/J mice at 36 weeks of age. Immunogold-labelling of annexin II (depicted by arrows) can be detected solely in the mesangial area in control mice, whereas annexin II was present in mesangial cells, within the MM and GBM, and in electron-dense deposits (denoted by asterisk) in the GBM of NZBWF1/J. Annexin II labelling was also observed along the cell surface of mesangial cells (depicted by orange arrows); E, endothelial cell; FP, foot process; M, mesangial cell; P, podocyte. Boxed areas are enlarged to allow comparison of annexin II immunogold labelling in the mesangial area and GBM in control and NZBWF1/J mice. Scale bar = 1 and 2 μm in images from control and NZBWF1/J mice respectively.

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DOI: 10.1042/CS20160732

Author contribution

Study conception and design: S.Y. and T.M.C.; acquisition of data: K.F.C., M.K.M.C. and K.W.C.; retrieval of clinical data: D.Y.H.Y. and C.K.L.; analysis and interpretation of data: K.F.C., S.Y., K.W.C., C.S.O.T. and T.M.C.; drafting the article: K.F.C., S.Y. and T.M.C.; approval of the final version for submission: K.F.C., S.Y., M.K.M.C., D.Y.H.Y., K.W.C., C.K.L., C.S.O.T. and T.M.C.

Acknowledgements

We thank Mr W.S. Lee and Mr B. Yau at the Electron Microscopy Unit, the University of Hong Kong for their assistance in transmission electron microscopy work. We thank Mr Andrew Yim and Mr Chris Ng for their technical assistance.

Funding

This study was supported by the Hong Kong Research Grants Council General Research Fund [grant numbers HKU 760309M and HKU 760712M]; the University Merit Award; the University of Hong Kong Small Project Funding [grant number 201309176187]; UGC Matching Grant Schemes (Phases V and VI), the Estate of the late Mr. Chan Wing Hei, and a kind donation from Mr G. King. S.Y. is supported by the Wai Hung Charitable Foundation Limited and the 'Yu Chiu Kwong Chair in Medicine' Endowment Fund awarded to T.M.C.

Competing interests

The authors declare that there are no competing interests associated with the manuscript.

Abbreviations

Ab, antibody; APS, antiphospholipid syndrome; C3, complement 3; CSF-1R, colony stimulating factor 1 receptor; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; GBM, glomerular basement membrane; HBSS, Hank's buffered saline solution; Ig, immunoglobulin; NZBWF1, New Zealand Black and White F1 mice; OD, optical density; PBST, PBS containing 0.05% Tween-20; S.D., standard deviation; SLE, systemic lupus erythematosus; SLEDAI, systemic lupus erythematosus disease activity index; TEM, transmission electron microscopy.

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